THE PATHOGENICITY OF BACILLUS INFLUENZAE FOR LABORATORY ANIMALS

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INTRODUCTION

The severity and extent of the recent pandemic of influenza has stimulated many investigations of the etiology of this disease.

During the interval between the pandemic which started in 1889 and the one which began in 1918, the bacillus discovered by Pfeiffer[1] in 1892 was accepted rather generally as the cause of influenza. The etiologic relationship was questioned by some because the bacillus was frequently found in the normal throat and in connection with a number of other inflammatory conditions of the respiratory system. The finding of it under such conditions was assumed by some to be due to a diminution or loss, for the time being, of its virulence.

When influenza first appeared in extensive epidemic form in Europe in 1918, many bacteriologists were able to isolate the Pfeiffer bacillus from only a rather small proportion of the cases.[2] The same has also rather generally been true in this country. It is, therefore, the opinion of many that this organism is not the cause of the epidemic disease influenza.

On the other hand, the finding of this bacillus as the only organism in the lungs of many cases of influenza,[3] as well as in the spinal fluid of a number of cases of meningitis, indicates that it has pathogenic properties.

Our knowledge as to how the Pfeiffer bacillus causes disease is as yet rather meager. The data obtained by experiments on lower animals have been rather conflicting. It was with the idea of determining more definitely what effect this bacillus has on laboratory animals and how such action is produced, that our investigation was undertaken.

EXPERIMENTS

Source of Cultures.—We worked with two cultures. One represented the only organism found in the cerebrospinal fluid of a case of meningitis which terminated fatally. The other represented a mixture of four different cultures

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[1] Deutsch. med. Wchnschr., 1892, 18, p. 28.
[2] Jour. Am. Med. Assn., 1918, 71, p. 1573.
[3] Ibid., p. 1031.

kindly supplied to us by Dr. F. O. Tonney of the Chicago Health Dept. These were isolated from sputum during the recent epidemic of influenza. The organisms used were small nonmotile, gram-negative, nonspore-bearing aerobic bacilli which grew only on mediums that contained hemoglobin. The bacilli of young cultures had rounded ends and were well stained by fuchsin, but only slightly by methylene blue. Many showed bipolar staining. The colonies on oleate-hemoglobin agar were minute and discrete. The material used consisted of:

(a) Growths on oleate-hemoglobin agar[4] after incubation at 37 C. for 24 hours suspended in normal salt solution. This medium, which yields a more profuse growth than ordinary blood agar does, is prepared as follows:

1. Prepare meat infusion agar (2%) having a reaction of 0.3-0.5% acid to phenolphthalein. Sterilize, 94 parts. (Liquefy before adding the ingredient named below.)
2. Add a 2% solution of sodium oleate which has been made up with distilled water and sterilized in the autoclave, 5 parts.
3. Add to the foregoing while still hot, a suspension of red blood corpuscles prepared as follows: Centrifuge sterile defibrinated human or rabbit blood; pipet off the supernatant serum; make up to original volume by the addition of sterile broth, 1 part.
4. Place in slanting position until solidified.

(b) Growths in oleate-hemoglobin broth, after incubation at 37 C. for 24 hours. This medium is prepared in the same way as the corresponding agar preparation except that meat infusion broth is substituted for the agar.

(c) Toxin of B. influenzae produced in oleate-hemoglobin broth and obtained by the following method:

1. Oleate-hemoglobin broth is inoculated heavily with a 24-hour culture of B. influenzae grown on oleate-hemoglobin agar.
2. Incubate for 24 hours at 37 C.
3. Centrifugate to throw down coarse particles.
4. Filter through a Mandler candle filter.

NOTE.—Use as soon after filtration as possible, since the toxin rapidly deteriorates.

(d) Toxin of B. influenzae produced in veal infusion blood broth and obtained by the following method suggested by Parker:[5]

1. Prepare a veal infusion broth neutral to phenolphthalein.
2. To 50 c.c. of this broth in an Erlenmeyer flask, add 5 c.c. of sterile defibrinated rabbit's blood.
3. Heat the mixture over a water bath at 75 C. until the blood coagulates and settles on standing. (This requires from 3-5 minutes after this temperature has been reached.)
4. Inoculate each flask with one or two slants of B. influenzae grown on oleate-hemoglobin agar.
5. Incubate for 24 hours at 37 C.
6. Centrifugate to remove coarse particles.
7. Filter through Mandler candle filter.

[4] Ibid., p. 2050.
[5] Ibid., 1919, 72, p. 476.

The animals used were rabbits, guinea-pigs and white mice. Animals varying in age and size were chosen. Inoculations were subcutaneous, intraperitoneal and into the blood.

Results of Inoculations.—The more significant data in connection with the inoculations and the results obtained are included in the tables.

The inoculations were followed, as a rule, within an hour — sometimes within half an hour — at other times, several hours, by indisposition. The symptoms were listlessness; loss of appetite; ruffling of the fur; rapid breathing; dyspnea; fever, and frequently also, twitching of muscles; convulsions; tonic spasms, resulting in retraction of the head; muscular weakness and loss of weight resulting, at times, in marked emaciation.

If the animal continued to live, the more acute symptoms usually passed off after two or three days. On the other hand, in spite of a reasonably good appetite, loss of weight and marked muscular weakness often continued for several weeks, after which some of the animals recovered, while others succumbed. Shortly before death the temperature would usually go below normal. Death, whether early or late, was usually preceded by convulsions. Fatal results occurred in from 1½ hours to 3 weeks. When the injections were made intraperitoneally, a peritonitis with marked tenderness of the abdomen frequently resulted. Some of the more significant records follow:

1. RABBIT 13.—Weight 517 gm., received an intravenous injection of 2 c c of a 24-hour oleate-hemoglobin broth culture from the "meningitis" strain of B. influenzae. Forty minutes later, the rabbit looked sick, was limp and respirations were deep and labored. In another 30 minutes, the animal was very weak, the head was retracted and presented jerky spasmodic movements. At this time there were also marked convulsions — the whole body shaking with clonic spasms. Breathing was rapid. Twitching of muscles accompanied by occasional convulsions and retraction of the head continued and became progressively worse for another two hours, at the end of which the animal died — three hours after the injection.

This is an example of the acute effects of the Pfeiffer bacillus as seen in a number of the younger rabbits. The symptoms are the same as produced in cases injected with the "toxin" alone. The effects are evidently the results of intoxication rather than infection. The strain of B. influenzae recovered from sputum produced similar effects.

2. MOUSE 15.—Received an intraperitoneal injection of 2 c c of an oleate-hemoglobin broth culture of the B. influenzae, isolated from the case of meningitis. Death occurred in 10 hours. A small amount of fluid was found in the peritoneal cavity. The Pfeiffer bacillus was isolated from the peritoneal fluid and the heart blood. Another bacillus which grew on plain agar was also isolated from the heart blood.

This is an example of the acute effects of the influenza bacillus. The presence of the bacillus in the blood indicates its power to invade or pass through tissue. The other organism found in the blood belonged to the colon group and was presumably derived from the intestinal tract of the mouse. Its invasion of the tissue was probably due to the lowered resistance caused by the injection of the influenza bacillus.

3. GUINEA-PIG 10.—Received an intraperitoneal injection of 5 c c of the toxin of the "meningitis" strain of B. influenzae grown in veal infusion blood broth. The temperature of the animal just before inoculation was 101.8 F.; the next day it was 104.2 degrees. It remained at about this point for 3 days, during which time the animal ate but little. After this time the appetite improved and

the temperature went down to 102 degrees. By the end of the seventh day, the weight had dropped from 597 to 440 gm. — a loss of 157 gm. He then received an intracardial injection of 5 c c of an oleate-hemoglobin broth culture. An hour later he was quite sick, and death occurred about 10 hours after injection. No B. influenzae were found, but another organism which grew on plain agar was obtained from the heart blood.

This experiment shows the acute and subacute effect of the "toxin" — characterized by indisposition, fever, loss of appetite and weight. The animal was on the road to recovery when the second injection was given. This produced acute symptoms. The presence of the organism recovered can no doubt be explained on the same basis as that given in connection with Mouse 15.

The more significant data of the more important experiments have been arranged in three tables. Each table represents the experiments for a different kind of animal. The nature of the material injected has already been explained. The letter "C" indicates that the material used was a mixture of four cultures obtained from Chicago through Dr. Tonney. In all other instances, the organism used was the one isolated from a case of Pfeiffer bacillus meningitis.

TABLE 1

EFFECT OF PFEIFFER BACILLUS ON RABBITS

Number Used	Size	Material Injected	Amount	Method	Death in	Remarks
3	Small	Veal broth	8 c c	Intravenous	.....	No effect. Control
4	Medium to large	Oleate broth	3-15 c c	Intravenous	.....	No effect. Control
3	Small	Oleate agar culture	½ tube in 3 c c salt solution	Subcutaneous	3 hours (one)	All sick
4	Small	Oleate agar culture	1 tube in 5 c c salt solution	Intravenous	7 hours 12 hours 30 hours	Pfeiffer bacillus obtained from heart blood Pfeiffer bacillus obtained from heart blood One recovered but became weak and lost 135 gm. in 19 days
4	Small	Oleate broth culture	1-3 c c	Intravenous	1¾ hours 3 hours 12 hours	Pfeiffer bacillus obtained from heart blood Pfeiffer bacillus obtained from heart blood One recovered
7	Small to medium	Oleate broth culture	6-8 c c	Intravenous	6 days 18 days 3 weeks	Four recovered Foul smelling organism isolated Had marked meningeal symptoms
1	Small	Oleate broth culture	5 c c	Intraperitoneal	.....	Recovered
1	Adult	Toxin-veal broth	4 c c	Subcutaneous	1½ hours	Several convulsions and rapid breathing
3	Adult	Toxin-veal broth	5-8 c c	Intraperitoneal	.....	Very sick but recovered
5	Adult	Toxin-oleate broth	4-8 c c	Intravenous	1½ and 1¾ hours	Three recovered
3	Half	Toxin-oleate broth	6 c c	Intravenous	5 hours 4 weeks	Marked convulsions One recovered

TABLE 2

EFFECT OF PFEIFFER BACILLUS ON GUINEA-PIGS

Number Used	Material Injected	Amount	Method	Death in	Remarks
1	Oleate broth	5 c c	Intracardial	.....	No effect. Control
2	Oleate broth culture	5 c c	Intracardial	10 and 19 hours	Pfeiffer bacillus obtained from the heart blood of one
1	Oleate broth culture	5 c c	Intracardial	19 hours	Pfeiffer bacillus recovered from heart blood
1	Toxin-veal broth	2 c c	Intracardial	.....	Recovered
2	Toxin-veal broth	5 c c	Intracardial	3 days	Lost 99 gm. in weight; one recovered
1	Oleate agar culture	⅙ tube in 1 c c salt solution	Subcutaneous	.....	Lost 134 gm. in weight
2	Oleate agar culture	1 tube in 5 c c salt solution	Intracardial	12 hours each	Another organism was isolated from heart blood of each
2	Oleate agar culture	1 tube in 5 c c salt solution	Intraperitoneal	23 and 48 hours	Another organism was isolated from heart blood of each

TABLE 3

EFFECT OF PFEIFFER BACILLUS ON MICE

Number Used	Material Injected	Amount	Method	Death in	Remarks
4	Oleate broth	2 c c	Subcutaneous (2), Intraperitoneal (2)	.....	No effect. Control
4	Oleate broth	2 c c	Subcutaneous	9 hours 24 hours 3 days	One recovered; another organism obtained from heart blood of two
3	Oleate broth	2 c c	Intraperitoneal	8 hours 9 hours 24 hours	Another organism obtained from heart blood of one
2	Toxin-veal broth	2 c c	Intraperitoneal Subcutaneous	24 hours 10 days	Another organism obtained from heart blood of each
3	Toxin-oleate broth	2 c c	Subcutaneous	8 days	Two recovered
3	Toxin-oleate broth	2 c c	Intraperitoneal	2 days	Two recovered
2	Oleate agar culture	1/10 tube in ½ c c salt solution	Intraperitoneal	3 days each	
2	Oleate agar culture	½ tube in 2 c c salt solution	Subcutaneous	22 hours and 4 days	
1	Oleate agar culture	½ tube in 2 c c salt solution	Intraperitoneal	48 hours	
2	Oleate agar culture and another organism	½ tube in 2 c c salt solution	Intraperitoneal	12 and 48 hours	The "contaminating" organism recovered from peritoneal fluid
1	Oleate agar culture and another organism	½ tube in 2 c c salt solution	Subcutaneous	21 hours	The "contaminating" organism recovered from heart blood

DISCUSSION

Our and to a certain extent also other experiments have shown that rabbits, guinea-pigs and mice are all susceptible to the Pfeiffer influenza bacillus. Other experiments have shown that monkeys[6] and, to a slight extent, dogs,[7] are also susceptible.

Rabbits.—Pfeiffer[8] demonstrated as early as 1893 that rabbits were susceptible to this organism. As symptoms he emphasized dyspnea and muscle weakness. Delius and Kolle[7] and Cantani[9] have also demonstrated the pathogenicity of B. influenzae to rabbits. They mention especially the emaciation as a manifestation of the chronic cases. Of 32 rabbits injected by us with a culture or the "toxin" of the influenza bacillus, 15, or 46.8%, succumbed in from 1½ hours to 3 weeks after injection. The remainder were ill for a variable length of time.

Guinea-Pigs.—Of 10 guinea-pigs injected, 7, or 70%, succumbed in from 10 hours to 3 days. All of the pigs showed some signs of indisposition. Guinea-pigs appear to be more uniformly susceptible to the influenza bacilli than rabbits, although the higher mortality may be explained in part by the relatively larger doses injected. None of the pigs succumbed as promptly as did some of the rabbits. The susceptibility of guinea-pigs has previously been shown by Delius and Kolle[7] and also by Kikuchi.[10]

Mice.—Of 24 mice injected by us, all but 4, or 83.3%, succumbed. This is in rather marked contrast with the recent statement of Spooner, Scott and Heath,[11] to the effect that "more than a hundred intraperitoneal injections of mice have shown that the organism is not pathogenic to that animal." On the other hand, Jacobsohn[12] succeeded in recovering influenza bacilli from the blood of mice provided they at the same time received an injection of dead streptococci or pneumococci.

The difference in the results obtained by different investigators is evidently due to a difference in the virulence of the organism and possibly also to the size of the dose.

Monkeys.—Pfeiffer and Beck[6] succeeded in inducing coryza in a monkey by rubbing a pure culture on the unbroken mucous membrane

[6] Deutsch. med. Wchnschr., 1892, 18, p. 465.
[7] Ztschr. f. Hyg. u. Infectionskrankh., 1897, 24, p. 327.
[8] Ibid., 1893, 13, p. 357.
[9] Ibid., 1896, 23, p. 265.
[10] Nippon-Eiseigakkwai-Zasshi, 1909, 3.
[11] Jour. Am. Med. Assn., 1919, 72, p. 155.
[12] Arch. de méd. expér., 1901, 13, p. 425.

of the nose. The symptoms were suggestive, but not at all typical of influenza in man. The introduction into the lungs of a suspension of the micro-organism into one monkey resulted in the development of fever and an illness which lasted for several days.

Wollstein[13] succeeded in producing a meningitis in monkeys by the intraspinal injection of pure cultures of virulent strains of B. influenzae.

Dogs.—Delius and Kolle[7] found that the subcutaneous or intraperitoneal injection of 50-60 agar cultures had no effect on dogs and that intravenous injections produced but slight symptoms. Larger doses, however, as also intracerebral injections of smaller ones, produced symptoms of intoxication.

We found that young (half-grown) rabbits and guinea-pigs were more susceptible than older ones. Delius and Kolle[7] also found that small guinea-pigs could be infected and killed by cultures of the organism to which larger pigs were resistant. This fact, together with the difference in virulence and dosage, may account for the fact that Pfeiffer[8] and more recently, Spooner, Scott and Heath[11] were unable to demonstrate any pathogenicity of this organism to guinea-pigs.

The route by which cultures or the "toxins" of the Pfeiffer bacillus were introduced into the body did not make as much difference as was to be expected. We found that in mice, the subcutaneous method produced results almost as quickly as the intraperitoneal method. In rabbits likewise there was no noticeable difference between subcutaneous, intraperitoneal and intravenous injections. Delius and Kolle[7] found that guinea-pigs succumbed to ¼ of an agar culture when given intraperitoneally although it required the entire growth to produce a fatal result when introduced subcutaneously. The few intratracheal insufflations which we made were negative. Positive results in rabbits have, however, been obtained in this manner by Pfeiffer[8] and by Spooner, Scott and Heath.[11]

Pfeiffer[8] succeeded in producing an inflammation by simply rubbing pure cultures on the unbroken nasal mucous membrane of a monkey and a rabbit. Delius and Kolle[7] found that dogs were most susceptible when the bacteria were introduced intracerebrally, but slightly affected by intravenous, and still less so by subcutaneous and intraperitoneal injections.

[13] Jour. Exper. Med., 1911, 14, p. 73.

We found that 2 c.c. of a broth culture or ½ of an agar culture suspended in 2 c c of salt solution was usually fatal to mice. This is a large dose for such a small animal. Controls consisting of an equal amount of the sterile fluid medium did not produce any harmful effects. Five c c of a broth culture was usually fatal to both guinea-pigs and rabbits. The controls were invariably negative even when as much as 8 and 15 c c of the sterile broth were introduced into the ear vein of rabbits. In some instances 1, 2 and 3 c c doses of the broth cultures and ½ of an agar culture were fatal to rabbits.

Various experimenters have found a marked difference in the virulence of influenza bacilli obtained from different sources. Some appear to be avirulent. Wollstein[13] found that cultures of this organism from the meninges are distinctly more virulent to rabbits than are those from sputum. Our experiments tend to confirm this.

Kikuchi[10] reports that he was able to increase the virulence of this bacillus by successive passage of the organism from one guinea-pig to another, using the peritoneal fluid of the succumbed animal for making the inoculations. The virulence was increased to the extent that whereas at first it required 8 agar cultures to produce the death of a young guinea-pig, after 6 successive inoculations, an injection of only $\frac{1}{10}$ of a c c of the peritoneal exudate was sufficient to kill the animal.

The injection of influenza bacilli or their "toxins" often resulted in the invasion of the tissues by another organism. This was no doubt due to the lowering of vitality produced by the influenza bacillus. We recovered two other organisms in this way. Injections of a mixture of the influenza bacillus and the other organism were more rapidly fatal than injections of the influenza bacillus alone.

Jacobsohn[12] was able to produce an influenza bacillus bacteriemia in mice only when using an impure culture of the organism.

Pfeiffer[8] and also Delius and Kolle[7] believed that the bacteria introduced intravenously were rapidly destroyed. Although we recovered influenza bacilli only from animals that succumbed within 30 hours after injection, we believe that the continuation of symptoms, especially loss of weight, weakness and convulsions can be explained on the basis that living organisms are present and continue to produce toxins. It is, of course, quite possible that the more chronic manifestations observed were due to the action of secondary invaders.

Pfeiffer[8] found no evidence that the bacteria increased in number in the body. Delius and Kolle[7] did not find any increase when the bacteria were injected intravenously into rabbits, but did find that they increased in number when introduced intraperitoneally into rabbits, guinea-pigs and mice, and intracerebrally in dogs.

We recovered the Pfeiffer bacillus from the heart blood after intraperitoneal injections—three times—once from each kind of animal used in the experiments. Pfeiffer[8] was unable to produce a bacteriemia in rabbits when the bacteria were not introduced into the veins.

Cantani[9] was unable to demonstrate invasion of tissue following subdural inoculations. On the other hand, Jacobsohn[12] succeeded in producing an influenza bacillus bacteriemia in mice if at the same time these animals received an injection of killed streptococci or pneumococci. Neither he nor Saathoff[14] were able to isolate the influenza bacilli from the blood of mice when only pure cultures were used. Evidently the invasive power of the influenza bacillus depends, not only on the virulence of the organism as in our case, but also on the lowering of the resistance of the body by other bacteria.

The production of pronounced symptoms within a few hours after inoculation must be due to a marked, often profound, intoxication. Pfeiffer[8] and other earlier investigators believed that this was due to an endotoxin liberated as the result of the rapid destruction of the bacilli in the body. Pfeiffer noticed no difference in the results when rabbits were inoculated with living bacteria or with cultures killed by chloroform. He believed therefore that it was not a case of infection but rather of intoxication.

Delius and Kolle[7] came to practically the same conclusion. They also found that the filtrate of killed cultures was toxic, although through instability, its toxicity was rapidly lost. Cantani[9] demonstrated that cultures killed by heating to 60 C. were fatal to guinea-pigs when injected either intracerebrally or intraperitoneally.

Slatineau[15] obtained what he regarded as the endotoxin by the following method: A suspension of an agar culture in salt solution was centrifugated. The sediment was treated with equal parts of fresh horse serum and distilled water. This was left in the incubator for 12 hours and again centrifugated. The supernatant fluid, supposed to contain the endotoxin liberated through the destruction of

[14] München. med. Wchnschr., 1901, 54, p. 2220.
[15] Centralbl. f. Bakteriol., I, O., 1906, 41, p. 185.

the bacteria by the serum water, was found to be toxic to guinea-pigs. He also found that the bacteria themselves after the treatment referred to were still toxic.

Parker[5] succeeded in obtaining a poison produced by the influenza bacillus by filtering veal infusion blood broth cultures after a period of incubation of from 6-20 hours. Two c c of the poison killed a medium sized rabbit in from 1-3 hours. The poison deteriorated very rapidly even when kept in an icebox. She was also able to make rabbits immune to this poison. The immune serum appeared to be antitoxic in nature.

We have been able to confirm Parker's experiments with reference to the production of a filtrable poison. An equally effective poison was produced in oleate-hemoglobin broth. We believe the production of the poison to be too rapid to be explained on the basis of an endotoxin alone. Death of the animal was produced, as a rule, more promptly when using broth cultures than when using an equal volume of the filtrates. From the fact that agar cultures suspended in salt solution were almost as effective in producing symptoms and death as a broth culture of corresponding size, we conclude that the disease is more of an infection than was held to be the case by previous investigators and that toxins, chiefly extracellular, are produced in the body of the inoculated animal.

SUMMARY AND CONCLUSIONS

The Pfeiffer influenza bacillus is distinctly pathogenic to mice, guinea-pigs and rabbits. This quality is apparently limited to certain cultures or strains of this micro-organism.

The guinea-pig is more uniformly susceptible to the influenza bacillus than rabbits and mice, although not as susceptible as some rabbits. Rabbits show a greater variation in individual susceptibility.

Young (half-grown) animals are more susceptible than larger ones.

Intravenous and intraperitoneal injections are slightly more rapidly fatal than subcutaneous injections.

Two c c of a 24-hour hemoglobin broth culture of a virulent organism is fatal to about 90% of white mice. Five c c of such a culture is fatal to about 50% of rabbits and 70% of guinea-pigs.

Death of animals occurs in from 1½ hours to 30 days after injection. It is probable that deaths occurring after the fourth or fifth days may be caused by secondary invading organisms rather than by the Pfeiffer bacillus.

The chief acute symptoms are listlessness, muscular weakness, rapid and labored breathing, elevation of temperature and convulsions. The chief chronic symptoms are loss of weight and muscular weakness.

The virulence of the organism varies with cultures from different sources.

The injection of influenza bacilli favors the invasion of tissue by other bacteria. Likewise the introduction of other bacteria favors the proliferation in the body and the invasion of tissues by the influenza bacillus.

Influenza bacilli are, as a rule, apparently rapidly destroyed soon after introduction into the body. Following injection into the peritoneal cavity they may appear in the blood. This appears to be dependent on either the virulence of the micro-organism or a condition of lowered resistance on the part of the body.

The influenza bacillus produces a toxin which is fatal to mice, guinea-pigs and rabbits almost as rapidly as are broth cultures of equal dosage. This toxin is produced very rapidly and can be obtained by filtering broth cultures. It is not possible to state definitely whether it is an endotoxin or an extracellular one.

Although the symptoms of intoxication as seen in lower animals following injections of the Pfeiffer bacillus are suggestive of the profound intoxication seen in connection with many cases of the epidemic disease influenza in the human being, these experiments do not furnish any proof that the Pfeiffer bacillus has any specific etiologic relationship to that disease. On the other hand, they suggest that a possible etiologic relationship cannot be ignored.